

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 1 108 743 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
20.06.2001 Bulletin 2001/25

(51) Int Cl.⁷: **C08K 3/00, C12N 7/00**

(21) Application number: **00126977.8**

(22) Date of filing: **08.12.2000**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **08.12.1999 JP 34904199**
16.02.2000 JP 2000038010

(71) Applicant: **JSR Corporation**
Tokyo (JP)

(72) Inventors:
• **Sato, Kouei**
Sashima-gun, Ibaraki-ken (JP)
• **Tanaka, Takeshi**
Tokyo (JP)

- **Murata, Mitsuhiro**
Ushiku-shi, Ibaraki-ken (JP)
- **Nishida, Shozou**
Iizuka-shi, Fukuoka-ken (JP)
- **Hikata, Mikio**
Tsuchiura-shi, Ibaraki-ken (JP)
- **Kasai, Kiyoshi**
Kameyama-shi, Mie-ken (JP)

(74) Representative:
Leson, Thomas Johannes Alois, Dipl.-Ing. et al
Patentanwälte
Tiedtke-Bühling-Kinne & Partner,
Bavariaring 4
80336 München (DE)

(54) **Virus-binding particles, virus-separating reagent, separation of viruses, and detection of viruses**

(57) Particles capable of being bound by viruses, having a particle diameter of 0.05 μm to 300 μm and having either a cationic group or an anionic group or both at their surfaces. Also disclosed are a method of

separating or detecting viruses and a virus-separating reagent, which make use of such particles. The particles enable simultaneous and simple treatment of a large number of specimens by a simple means.

EP 1 108 743 A2

Description

BACKGROUND OF THE INVENTION1. Field of the Invention

[0001] This invention relates to virus-binding particles for separating viruses present in samples, and a virus-separating reagent, a method of separating viruses and a method of detecting viruses using the same.

2. Description of the Prior Art

[0002] Viruses may cause various diseases of human beings, animals and vegetables. For detecting viruses to diagnose diseases, it is very important to ascertain causative viruses. As conventional methods for detecting viruses to make diagnosis, it is common to assay virus antigens or antiviral antibodies immunologically. Since, however, viruses or antiviral antibodies are in a small quantity for several weeks to several months after viral infection, the viruses can not be detected by such immunological assay in some cases. The period for which they can not be detected is called a window period (blank period). If some patients standing in such a period donate blood, the blood thus donated may often be well infectious, and has a possibility of bringing many unspecified blood-transfused patients or blood preparation utilizer patients into harmful condition. Accordingly, in order to make the window period as short as possible, it is a pressing need to develop a technique by which any viruses standing below the limit of immunological detection can be detected at a high sensitivity.

[0003] In recent years, nucleic-acid amplification techniques as typified by the polymerase chain reaction process (hereinafter "PCR process") has brought about a possibility for enabling detection of viruses present even in a trace quantity. However, the PCR process and so forth also require special equipment and high-grade techniques for such detection of viruses present in a trace quantity, and it is very difficult to put it into practice with ordinary equipment and in a simple way. Accordingly, in order to solve such problems, methods of separating viruses present in a specimen are used.

[0004] As a typical example of conventional methods for separating viruses, ultracentrifugation is available, which, however, requires an expensive machinery and a long time and also can not make simultaneous treatment of a large number of specimens with ease, and is hard to regard as a simple method. Also reported is chromatography making use of a heparin-Sepharose carrier, utilizing the characteristic that a type B hepatitis virus surface antigen (HBs antigen) combines with heparin. It is also difficult for this method to make simultaneous treatment of a large number of specimens. As the other methods, also available are methods of causing viruses to sediment by a method making use of ammonium sulfate or combination of polyethylene glycol or polyanion with a divalent ion, e.g., a particulate substance having acidic groups (Japanese Post-examination Publication (Kokoku) No. 6-22627) or combination of particles with a divalent metal (Japanese Laid-open Publication (Kokai) No. 6-217767), or by a method in which a water soluble polymeric substance having cationic groups is added to remove viruses (Japanese Laid-open Publication (Kokai) No. 4-342536). These methods, however, involve a difficulty that samples must be purified after viruses have been separated by sedimentation, because of problems such that reagents to be mixed and proteins mixed in a large quantity in the viruses thus separated cause the inhibition of PCR.

SUMMARY OF THE INVENTION

[0005] Accordingly, an object of the present invention is to solve the above problems in virus separation methods to provide virus-binding particles which enable simultaneous and simple treatment of a large number of specimens by a simple means and which are readily adaptable to automation and do not adversely affect any nucleic-acid amplification tests, and to provide a virus-separating reagent, a virus separation method and a virus detection method which make use of such particles.

[0006] As a result of researches, as a means for solving the above problems, the present inventors have accomplished development of the following virus-binding particles and separation method.

[0007] Thus, firstly, the present invention provides particles capable of being bound by viruses, having a particle diameter of 0.05 μm to 300 μm and having either a cationic group or an anionic group or both at their surfaces. Said particles are hereinafter referred to "virus-binding particles".

[0008] Secondly, the present invention provides a method of separating viruses, comprising the steps of:

adding virus-binding particles to a sample which possibly contains viruses, to allow the viruses to bind to the particles; said virus-binding particles having a particle diameter of 0.05 μm to 300 μm and having either a cationic group or an anionic group or both at their surfaces; and

separating from the sample the particles to which the viruses have bound.

[0009] Thirdly, the present invention provides a method of detecting viruses, comprising the steps of:

5 adding virus-binding particles to a sample which possibly contains viruses, to allow the viruses to bind to the particles; said virus-binding particles having a particle diameter of 0.05 μm to 300 μm and having either a cationic group or an anionic group or both at their surfaces;
separating viruses from the sample by separating from the sample the particles to which the viruses have bound, to collect the viruses; and
10 subjecting the viruses thus separated, to a nucleic-acid amplification test.

[0010] Fourthly, the present invention also provides a virus-separating reagent comprising polymer particles having an anionic group at their surfaces and a polyvalent metal compound.

15 DETAILED DESCRIPTION OF THE INVENTION

[0011] The present invention will be described below in detail.

- Virus-binding particles -

20 [0012] In the present invention, virus-binding particles refer to particles which adsorb viruses present in blood or humors and from which the viruses having been adsorbed thereon are to be separated. The viruses having been separated by the particles are used to make nucleic-acid extraction, examination and diagnosis, in particular, examination and diagnosis involving nucleic-acid amplification. The virus-binding particles of the present invention have at
25 least one of a cationic group and an anionic group (both inclusive of the state of salts) at particle surfaces, and have a particle diameter of from 0.05 to 300 μm .

[0013] The virus-binding particles of the present invention may be any of water-insoluble materials without any particular limitations, and are comprised of particles formed of such materials, and at least one of a cationic group and an anionic group which is present at the surfaces of the particles.

30 [0014] The cationic group in the present invention may include an amino group, such as a primary amino group, a secondary amino group and a tertiary amino group, a quaternary ammonium group, an imino group such as a primary imino group, a secondary imino group and a tertiary imino group, a quaternary iminium group, an amidino group such as a primary amidino group, a secondary amidino group and a tertiary amidino group, a quaternary amidinium group,
35 a hydradino group such as a primary hydradino group, a secondary hydradino group and a tertiary hydradino group, a quaternary hydrazinium group, or an anionic nitrogen-containing cyclic group such as pyridyl groups and a quaternary pyridinium group.

[0015] In the present invention, the cationic group includes a group capable of combining with a proton such as an amino group to form a cation, and a group formed by the reaction of such a group with an acid to produce a salt in which a cationic moiety is formed by the group.

40 [0016] In the present invention, the cationic group may inhibit nucleic-acid amplification such as PCR if it dissolves out in water, buffer solutions, blood or humors, and hence must be chemically combined with the particles. It may be present in an amount of at least 1×10^{-10} mol, typically from 1×10^{-10} to 1×10^{-2} mol, preferably from 1×10^{-9} to 1×10^{-3} mol, and more preferably from 1×10^{-8} to 1×10^{-3} mol, per g of particles on the average. If the cationic group is present in an amount less than 1×10^{-10} mol, the particles may have an insufficient virus-separating ability. Though
45 the above upper limit is not critical, it is often difficult to introduce the cationic group in an amount more than 1×10^{-2} mol.

[0017] The particles having a cationic group can be produced by, e.g., (1) a process in which monomer components containing a cationic monomer are polymerized; (2) a process in which a monomer is polymerized in the presence of a radical polymerization initiator having a cationic group; and (3) a process in which a compound having a cationic group is made to combine with particles.
50

(1) Process in which monomer components containing a cationic monomer are polymerized:

[0018] In this process, usable cationic monomers may include, for example,

55 aminoalkyl group-containing (meth)acrylates ["(meth)acryl..." is meant to be acryl... or methacryl... or a mixture of these; the same applies hereinafter] such as 2-dimethylaminoethyl (meth)acrylate, 2-diethylaminoethyl (meth)acrylate, 2-dimethylaminopropyl (meth)acrylate and 3-dimethylaminopropyl (meth)acrylate, and quaternary salts of these with methylene chloride, dimethyl sulfate, diethyl sulfate or the like;

aminoalkoxyalkyl-group-containing (meth)acrylates such as 2-(dimethylaminoethoxy)ethyl (meth)acrylate, 2-(diethylaminoethoxy)ethyl (meth)acrylate and 3-(dimethylaminoethoxy)propyl (meth)acrylate, and quaternary salts of these with methylene chloride, dimethyl sulfate, diethyl sulfate or the like;

N-aminoalkyl-group-containing (meth)acrylamides such as N-(2-dimethylaminoethyl) (meth)acrylamide, N-(2-diethylaminoethyl) (meth)acrylamide, N-(2-dimethylaminopropyl) (meth)acrylamide and N-(3-dimethylaminopropyl) (meth)acrylamide, and quaternary salts of these with methylene chloride, dimethyl sulfate, diethyl sulfate or the like. In particular, 2-dimethylaminoethyl (meth)acrylate, N-(2-diethylaminoethyl) (meth)acrylamide, and quaternary salts of these with methylene chloride are preferred.

[0019] Any of these may be used singly or in combination of two or more.

[0020] The monomer copolymerizable with the cationic monomer may include crosslinkable monomers and non-crosslinkable and nonionic monomers as shown below.

[0021] The crosslinkable monomers may include, for example, divinyl monomers, trivinyl monomers and tetravinyl monomers, such as divinylbenzene, divinylbiphenyl, ethylene glycol di(meth)acrylate, diethylene glycol di(meth)acrylate, triethylene glycol di(meth)acrylate, tetraethylene glycol di(meth)acrylate, propylene glycol di(meth)acrylate, dipropylene glycol di(meth)acrylate, tripropylene glycol di(meth)acrylate, tetrapropylene glycol di(meth)acrylate, 1,4-butanediol di(meth)acrylate, 1,6-hexanediol di(meth)acrylate, neopentyl glycol di(meth)acrylate, 2,2'-bis[4-(meth)acryloyloxypropoxyphenyl]propane, 2,2'-bis[4-(meth)acryloyloxydiethoxydiphenyl]propane, glycerol tri(meth)acrylate, trimethylolpropane tri(meth)acrylate, and pentaerythritol tetra(meth)acrylate. In particular, divinylbenzene, ethylene glycol dimethacrylate and trimethylolpropane trimethacrylate are preferred.

[0022] Any of these may be used singly or in combination of two or more.

[0023] The copolymerizable non-crosslinkable and nonionic monomers are monomers which are copolymerizable with either the cationic monomer or the crosslinkable monomers or both and are non-crosslinkable and nonionic.

[0024] Such monomers may include aromatic vinyl monomers such as styrene, α -methylstyrene, p-methylstyrene and halogenated styrenes; unsaturated nitriles such as acrylonitrile; acrylates and methacrylates, such as methyl acrylate, methyl methacrylate, ethyl acrylate, ethyl methacrylate, butyl acrylate, butyl methacrylate, cyclohexyl methacrylate, 2-ethylhexyl acrylate, 2-ethylhexyl methacrylate, lauryl acrylate, lauryl methacrylate, glycidyl acrylate, glycidyl methacrylate, 2-hydroxyethyl acrylate and 2-hydroxyethyl methacrylate; diolefines such as butadiene and isoprene; vinyl carboxylate esters such as vinyl acetate; and vinyl or vinylidene chlorides such as vinyl chloride and vinylidene chloride. In particular, styrene, α -methylstyrene, acrylonitrile, methyl methacrylate, butyl methacrylate, 2-hydroxyethyl acrylate and cyclohexyl methacrylate are preferred.

[0025] Any of these monomers may be used singly or in combination of two or more.

[0026] Polymeric components containing the cationic monomer described above can be obtained by emulsion polymerization, suspension polymerization or the like in the presence of a polymerization initiator in an aqueous dispersion medium.

[0027] The polymerization initiator is exemplified by redox type water-soluble polymerization initiators such as persulfates, hydrogen peroxide-ferrous chloride, and cumene hydroperoxide-sodium ascorbate; and oil-soluble polymerization initiators such as benzoyl peroxide, lauroyl peroxide, t-butyl peroxy-2-ethylhexanoate, and azobisisobutyronitrile.

[0028] Surface-active agents, dispersion stabilizers and so forth may also optionally be used.

(2) Process making use of a radical polymerization initiator having a cationic group:

[0029] This radical polymerization initiator is one in the presence of which a polymer obtained by radical polymerization comes to have at its terminal a cationic group derived from the radical polymerization initiator.

[0030] Preferred radical polymerization initiators having a cationic group may include azobis type initiators having an amidino group, an imidino group or a pyridium group. Those having a 10-hour half-life temperature in the range of from 40 to 95°C are preferred because the polymerization can be carried out under mild conditions.

[0031] As preferred specific examples of such radical polymerization initiators having a cationic group, they may include the following:

2,2'-azobis(2-methyl-N-phenylpropionamidine) dihydrochloride (available as VA-545, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[N-(4-chlorophenyl)-2-methylpropionamidine] dihydrochloride (available as VA-546, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[N-(4-hydroxyphenyl)-2-methylpropionamidine] dihydrochloride (available as VA-548, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-methyl-N-(phenylmethyl)-propionamidine] dihydrochloride (available as VA-552, trade name, from

Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-methyl-N-(2-propenyl)propionamidine] dihydrochloride (available as VA-553, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis(2-methylpropionamidine) dihydrochloride (available as V-50, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[N-(2-hydroxyethyl)-2-methylpropionamidine] dihydrochloride (available as VA-558, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[N-(2-carboxyethyl)-2-methylpropionamidine] hydrate (available as VA-057, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-methyl-(5-methyl-2-imidazolin-2-yl)propane] dihydrochloride (available as VA-041, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (available as VA-044, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-(4,5,6,7-tetrahydro-1H-1,3-diazepin-2-yl)propane] dihydrochloride (available as VA-054, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-(3,4,5,6-tetrahydropyrimidin-2-yl)propane] dihydrochloride (available as VA-058, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis[2-(5-hydroxy-3,4,5,6-tetrahydropyrimidin-2-yl)propane] dihydrochloride (available as VA-059, trade name, from Wako Pure Chemical Industries, Ltd.);

2,2'-azobis{2-[1-(2-hydroxyethyl)-2-imidazolin-2-yl]propane} dihydrochloride (available as VA-060, trade name, from Wako Pure Chemical Industries, Ltd.); and

2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (available as VA-061, trade name, from Wako Pure Chemical Industries, Ltd.).

[0032] In particular, use of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (V-50), 2,2'-azobis[N-(2-carboxyethyl)-2-methylpropionamidine] hydrate (VA-057) or 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) is preferred.

[0033] The radical polymerization initiator having a cationic group may preferably be used in an amount of from 0.1 to 10 parts by weight, and more preferably from 0.5 to 5 parts by weight, based on 100 parts by weight of the total sum of monomers used in the polymerization. Its use in an amount less than 0.1 parts by weight, which is too little, may make the resultant particles low cationic, and its use in an amount more than 10 parts by weight, which is in excess, may make the polymerization unstable, undesirably.

(3) Process in which a compound having a cationic group is made to combine with particles:

[0034] A monomer having a functional group such as a carboxyl group, a hydroxyl group, an epoxy group, an amino group or an amide group is subjected to copolymerization or seed polymerization to produce a polymer having functional groups, and these functional groups are allowed to react as linking moieties, whereby the compound having a cationic group can be introduced to particle surfaces.

[0035] The monomer having a functional group for introducing the functional groups may include acrylic acid, methacrylic acid, crotonic acid, itaconic acid, fumaric acid, maleic acid, hydroxyethyl methacrylate, 2-hydroxyethyl acrylate, glycidyl methacrylate, acrylamide, methacrylamide, N-methylolmethacrylamide and N-isopropylacrylamide.

[0036] Cationic compounds to be reacted with functional groups introduced to particle surfaces as described above may include, e.g., polyamines derived from biological samples, such as putrescine, cadaverine, spermidine, spermine, 1,3-diaminopropane, cardine, homospermine, 3-aminopropyl cadaverine, norspermine, thermospermine and caldopentamine, and polymerization products of these; basic proteins classified into histone and protamine, and polymers of these; polyamine compounds classified into polyalkylamines and polyalkylimines such as polyvinylamine, polyallylamine, polyethyleneimine and polypropyleneimine; polyamino acids such as polylysine, polyarginine and polyhistidine; other synthetic, natural or semi-synthetic (inclusive of fermentation and gene recombination) macromolecules such as polydiethylaminoethyl methacrylate, polydimethylaminoethyl methacrylate, polyvinylpyridine quaternized products, polybrene, chitosan, glycol chitosan, methyl glycol chitosan and polydiallyldimethylammonium; diamines such as ethylenediamine, trimethylenediamine, tetramethylenediamine, pentamethylenediamine and hexamethylenediamine; and silane coupling agents such as 3-aminopropyltriethoxysilane. They may also include polymers obtained by copolymerizing the monomers having an amine compound described above.

[0037] The anionic group in the present invention may include a carboxyl group and a sulfonic acid group ($-SO_3H$). These anionic groups may be present in the state they have formed salts. In the present invention, the anionic group, made present in at least part of particles, and preferably at the surfaces of particles, may inhibit nucleic-acid amplification such as PCR if it dissolves out in water, buffer solutions, blood or humors, and hence must be chemically com-

5 bined with the particles. It may be present in an amount of at least 1×10^{-10} mol, typically from 1×10^{-10} to 1×10^{-2} mol, preferably from 1×10^{-9} to 1×10^{-3} mol, and more preferably from 1×10^{-8} to 1×10^{-3} mol, per 1 g of particles on the average. If the anionic group is present in an amount less than 1×10^{-10} mol, the particles may have an insufficient virus-separating ability. Though the above upper limit is not critical, it is often difficult to introduce the anionic group in an amount more than 1×10^{-2} mol.

10 **[0038]** Such particles may include, e.g., surface-sulfonated particles, carboxyl group-containing particles, particles comprising synthetic polymeric particles with which a sulfonic acid group-containing monomer and/or a carboxyl group-containing monomer has or have been seed-polymerized or graft-polymerized, hydrogel particles comprised of a sulfonic acid group-containing monomer and a water-soluble crosslinkable monomer, polyanionic compound-fixed particles, and anionic inorganic particles. Without limitation to these, any particles may be used as long as they have an anionic group at at least part of the surface.

15 **[0039]** As the surface-sulfonated particles, usable are, e.g., particles comprised of a polymer or copolymer of a polymeric compound having at least at the surface of a particle a functional group capable of being sulfonated as exemplified by a back-bone-chain or side-chain unsaturated double bond, an aromatic group, a primary or secondary amino group, a primary alkyl halide group, an aliphatic aldehyde, an aliphatic ketone, an aliphatic carboxylic acid, an aliphatic carboxylic anhydride residual group or a hydroxyl group present on the backbone or side chains and having been sulfonated at at least part of the surface to have a sulfonic acid group. As examples of the polymeric compound constituting the particles capable of being sulfonated at their surfaces, it may include addition polymerization type polymeric compounds such as polymers or copolymers of monomers capable of being sulfonated, as exemplified by styrene, α -methylstyrene, vinyl naphthalene, divinylbenzene, butadiene, isoprene and vinyl alcohol, and copolymers of any of these monomers with different polymerizable monomers; and condensation polymerization type polymeric compounds such as polycarbonates, polyesters, polyester ethers, polyaryl ethers, polyalkylene oxides, polysulfones, polyether sulfones, polyamides, polyimides, polyether imides, polyether ketones, polyurethanes, acetaldehyde condensation products of aromatic compounds, and polyethers.

25 **[0040]** The particles comprised of any of these polymeric compounds can be sulfonated by treating the particles with concentrated sulfuric acid, fuming sulfuric acid, sulfuric anhydride, a sulfuric anhydride-dioxane complex, a sulfuric anhydride-pyridine complex, chlorosulfonic acid or the like.

30 **[0041]** The carboxyl-containing particles may include polymer or copolymer particles of a carboxyl group-containing monomer. Here, the carboxyl group-containing monomer (hereinafter "carboxylic acid monomer") refers to a polymerizable monomer having in the molecule an addition-polymerizable unsaturated bond and a carboxyl group. As specific examples thereof, it may include acrylic acid, methacrylic acid, crotonic acid, itaconic acid, fumaric acid and maleic acid.

[0042] These polymer or copolymer particles can be synthesized by a conventional method of emulsion polymerization or suspension polymerization.

35 **[0043]** The particles comprising synthetic polymeric particles with which a sulfonic acid group-containing monomer (hereinafter "sulfonic acid monomer") and/or a carboxylic acid monomer has or have been seed-polymerized or graft-polymerized may include particles obtained by subjecting seed particles comprised of a synthetic macromolecule, to seed polymerization or copolymerization or graft polymerization or copolymerization together with a sulfonic acid monomer and/or a carboxylic acid monomer and further optionally with a different copolymerizable monomer other than these. Such particles can be synthesized by adding a monomer and a radical generator to seed particles dispersed in water containing a surface-active agent or in a water/polar solvent mixture, followed by reaction carried out at 50 to 40 100°C . Here, the seed particles comprised of a synthetic macromolecule may include polymer or copolymer particles of aromatic compounds containing a polymerizable double bond, such as styrene, α -methylstyrene, vinyltoluene and vinyl naphthalene; cyan compounds containing a polymerizable double bond, such as acrylonitrile, methacrylonitrile and vinylidene cyanide; polymerizable crosslinkable compounds such as divinylbenzene and ethylene glycol dimethacrylate; organic compounds containing a polymerizable double bond, such as vinyl chloride, vinylidene chloride, vinyl methyl ethyl ketone, vinyl methyl ether, vinyl acetate, vinyl formate, allyl acetate, methallyl acetate, acrylamide, methacrylamide, N-methylolmethacrylamide, N-isopropylacrylamide, glycidyl acrylate, glycidyl methacrylate, acrolein, methacrolein, allyl alcohol, 2-hydroxyethylacrylate, 2-hydroxyethylmethacrylate, 2-methoxyethyl acrylate, n-butyl acrylate, sec-butyl acrylate, isobutyl acrylate, t-butyl acrylate, n-butyl methacrylate, sec-butyl methacrylate, isobutyl methacrylate, t-butyl methacrylate, methyl acrylate, ethyl acrylate, n-propyl acrylate, iso-propyl acrylate, methyl methacrylate, ethyl methacrylate, n-propyl methacrylate and iso-propyl methacrylate; and polymerizable cyclic compounds such as ethylene oxide, propylene oxide, 2-methyltetrahydrofuran, styrene oxide, butylene oxide and glycidyl ether.

50 **[0044]** The sulfonic acid monomer may include isoprenesulfonic acid, ethylenesulfonic acid, vinylsulfonic acid, styrenesulfonic acid, α -methylstyrenesulfonic acid, 2-acrylamido-2-methylpropanesulfonic acid, sulfoethyl acrylate and sulfonated dicyclopentadiene.

55 **[0045]** As the different polymerizable monomer, it may include aliphatic diene compounds such as 1,3-butadiene and isoprene; aromatic compounds containing a polymerizable double bond, such as styrene, α -methylstyrene and vinyltoluene; alkyl (metha)acrylates such as methyl acrylate, ethyl acrylate, 2-ethylhexyl acrylate, methyl methacrylate,

ethyl methacrylate, 2-hydroxyethyl acrylate and 2-hydroxyethyl methacrylate; polymerizable cyan compounds such as acrylonitrile and methacrylonitrile; organic compounds containing a polymerizable double bond, such as vinyl chloride, vinylidene chloride, vinyl methyl ethyl ketone, vinyl methyl ether, vinyl acetate, allyl acetate, methallyl acetate, acrylamide, methacrylamide, N-methylolmethacrylamide, N-isopropylacrylamide, glycidyl acrylate, glycidyl methacrylate, acrolein, methacrolein and allyl alcohol; and polymerizable cyclic compounds such as ethylene oxide, propylene oxide, 2-methyltetrahydroxyfuran, styrene oxide, butylene oxide and glycidyl ether.

[0046] As the hydrogel particles comprised of a sulfonic acid monomer and a water-soluble crosslinkable monomer, usable are, e.g., hydrogel particles comprised of a polymer of the sulfonic acid monomer described above with a water-soluble crosslinkable monomer such as N,N'-methylenebisacrylamide. Such particles can be obtained by vigorously mixing an aqueous solution of the sulfonic acid monomer, the water-soluble crosslinkable monomer and a radical reaction initiator with a non-polar solvent to which a surface-active agent has been added, to form an water-in-oil type reverse micelle, followed by reaction carried out at 50 to 100°C.

[0047] The polyanionic compound-fixed particles may include particles comprising particles having a functional group such as an epoxy group, an amino group, an aldehyde group, a carboxyl group, a hydroxyl group or an acid chloride group on particle surfaces of which a polyanion having in the molecule a plurality of sulfonic acid groups and/or carboxyl groups has been supported directly or via a coupling agent or a spacer. Here, the polyanion may include, e.g., polymeric compounds obtained by polymerizing or copolymerizing a sulfonic acid group-containing monomer and/or a carboxylic acid monomer and further optionally a monomer other than these; tungstophosphoric acids; and polyphosphoric acids.

[0048] As the anionic inorganic particles, $\text{SiO}_2\text{-Al}_2\text{O}_3$ -based particles on which sulfonic acid groups have been introduced, such as activated clay or the like may preferably be used. Also, in the case of particulate materials of glass, silica, alumina, activated carbon and so forth, their surfaces may be coated with a polymer having a sulfonic acid group or a carboxyl group. Such a polymer may include polyacrylic acid, polymethacrylic acid, polystyrenesulfonic acid, and copolymers of a monomer which is a constituent unit of any of these polymers with a hydrophilic monomer. The hydrophilic monomer may include hydrophilic acrylate monomers and hydrophilic methacrylate monomers. Apart from this, in the case of glass or silica, the carboxyl group can be introduced by first introducing an amino group using a silane-coupling agent such as γ -aminopropyltriethoxysilane, followed by reaction with succinic anhydride or with succinic acid in the presence of carbodiimide. Also, the sulfonic acid group can be introduced by first introducing an aldehyde group by treatment with glutaldehyde often the treatment with the silane-coupling agent, followed by reaction thereof with 2-aminoethanesulfonic acid.

[0049] The virus-binding particles of the present invention may comprise a magnetic material in the interiors, or at the surfaces, of the particles.

[0050] This magnetic material may preferably be incorporated only in the interiors and be not laid bare to the surfaces. In the present invention, the incorporation of a magnetic material in the virus-binding particles enables collection of particles by the action of magnetism possessed by the particles, and makes it unnecessary to collect them by centrifugation or the like. This not only makes it possible to shorten examination time, but also makes it easy to deal with the automation of examination and diagnosis. Such a magnetic material may include, e.g., ferrites of various types such as triiron tetraoxide (Fe_3O_4) and γ -iron sesquioxide ($\gamma\text{-Fe}_2\text{O}_3$), or metals such as iron, manganese, cobalt and chromium or alloys of any of these metals, any of which may be used.

[0051] The magnetic material may be contained in an amount not less than 10% by weight, and particularly from 20 to 100% by weight, preferably 20 to 90% by weight, based on the total weight of the virus-binding particles. If it is in too small a quantity, no good magnetically separable properties may be attained in the virus-binding particles. As the result, in the method of separating viruses as described later, it takes fairly long time to separate the virus-binding particles from specimens such as blood and humors, and hence any high time efficiency is not obtainable in some cases, undesirably.

[0052] In order to incorporate the magnetic material in the virus-binding particles, the following methods may be employed.

(a) The magnetic material is dispersed in a polymerization component containing the cationic monomer or anionic monomer, and polymerization is carried out in that state. The polymerization may be carried out by a process such as conventional emulsion polymerization, suspension polymerization or dispersion polymerization.

(b) A polymerization component containing the cationic monomer or anionic monomer is polymerized to synthesize polymer particles, and thereafter magnetic material layers are formed on the particle surfaces.

(c) On the surfaces of the particles obtained in the above (a) or

(b), polymer layers comprised of a polymerization component containing the cationic monomer or anionic monomer are further formed.

[0053] In some cases, the virus-binding particles thus obtained contain in their dispersion medium an emulsifier, a dispersant, unreacted monomers, a water-soluble polymer, a polymerization initiator decomposition product and so

forth. These substances have a high possibility of turning to reaction inhibitors in the step of a nucleic-acid amplification test. Accordingly, they may preferably be removed from the dispersion medium of the virus-binding particles by, e.g., the method disclosed in Adv. Colloid Interface Sci., 81, 77-165 (1999).

5 (Polyvalent Metal Compound)

[0054] In the present invention, a polyvalent metal compound may be added to a sample together with the virus-binding particles, whereby viruses can be made to bind to the virus-binding particles at a higher proportion in some cases.

10 **[0055]** Here, the polyvalent metal may include, e.g., Be, Mg, Sr, Ba, Ti, Zr, Cr, Mo, W, Mn, Fe, Ru, Os, Co, Rh, Ni, Pd, Pt, Cu, Ag, Au, Zn, Cd, Hg, Al, Ga, Si, Ge, Sn, Pb, P, As, Sb and Bi. The polyvalent metal compound refers to a compound capable of forming divalent or higher-valent cations upon dissociation in water, among chlorides, hydroxides, carbonic acid compounds, sulfuric acid compounds, nitric acid compounds, acetic acid compounds and chloric acid compounds of these polyvalent metals. Of these polyvalent metal compounds, magnesium chloride and the like are preferred.

15 **[0056]** The polyvalent metal compound may be used in such a quantity that it comes to be usually in a concentration of from 0.1 to 100 mmol/L in a reaction mixture formed when the virus-binding particles and a sample are mixed.

20 - Virus-separating Reagent -

[0057] In the present invention, the virus-separating reagent comprises the above virus-binding particles and the above polyvalent metal compound.

25 **[0058]** The preparation method of the reagent is not limited. The virus-separating reagent may be in the form the polyvalent metal compound has been added in a dispersion prepared by dispersing the virus-binding particles in an aqueous medium, or in the form the dispersion and the polyvalent metal compound are stored separately so that they are mixed immediately before use.

30 **[0059]** Any viruses in a specimen such as plasma or serum are separated in a high efficiency, and hence, usually, the virus-binding particles are used not in column chromatography but in a batch process. Accordingly, the particles may usually have particle diameters of from 0.05 μm to 300 μm , preferably from 0.1 μm to 100 μm , and more preferably from 0.2 μm to 80 μm . As long as the particles have particle diameters within this range, they are usable for what is intended in the present invention. Particles having particle diameters smaller than 0.05 μm are undesirable because such particles may make it necessary to carry out centrifugation at a larger number of revolutions or for a longer time of revolution when the virus-bound particles are separated from blood or a humor, so that the apparatus must be made larger in size or any high time efficiency can not be achieved. Particles having particle diameters larger than 300 μm are also undesirable because such particles may have a low efficiency for capturing viruses, and may make it unable in some cases to separate viruses well. Also, with regard to the particle shape of the virus-binding particles of the present invention, the particles need not be spherical, and may be irregular-shaped particles. As for the particle diameter of particles which are not spherical, an average value of the largest lengths and smallest breadths of individual particles is found.

40 - Separation of Viruses -

[0060] The method of separating viruses according to the present invention comprises the steps of:

45 adding the above virus-binding particles to a sample which possibly contains viruses, to allow the viruses to bind to the particles; and
separating from the sample the particles to which the viruses have bound, to collect the viruses.

[0061] A method of separating viruses by means of the virus-separating reagent of the present invention will specifically be described below.

50 **[0062]** The virus-separating reagent of the present invention has the ability to separate viruses of various types. For example, it can separate hepadnaviruses (such as hepatitis B virus), adenoviruses, flaviviruses (such as Japanese B encephalitis virus), herpesviruses (such as herpes simplex virus, varicella-zoster virus, cytomegalovirus, or EB virus), poxviruses, parvoviruses (such as adeno-related virus), orthomyxoviruses (such as influenza virus), rhabdoviruses (such as rabies virus), retroviruses (such as human immunodeficiency viruses) and hepatitis C virus.

55 **[0063]** Specimens for which the separation of viruses by the virus-separating reagent of the present invention is intended may include humors such as plasma, serum, cell lysate, urea and saliva, and cultured cell fragment fluid. Such specimens may be used as samples as they are, or may be used as samples after they have been diluted for

some purposes.

[0064] The virus-binding particles according to the present invention is desirably added to a sample in the form of a virus-separating reagent prepared by the virus-binding particles in a medium such as saline. The quantity in which the virus-separating reagent of the present invention is added in a sample depends on the concentration of viruses present in the sample. It may be so added that the virus-binding particles in the virus-separating reagent are usually in an amount of from 0.05 to 50% by weight, and preferably from 0.1 to 20% by weight, of the weight of the sample. Its addition in too small a quantity puts a limitation on the number of viruses which can bind to the virus-binding particles, resulting in a poor separation efficiency. Also, its addition in too large a quantity makes it necessary to use a detachment solution in a large quantity when bound viruses are detached in a post stage, resulting in a low separation efficiency.

[0065] Virus-binding particles which have adsorbed thereon the viruses in a sample are separated from the sample by centrifugation or natural sedimentation or, in the case when the virus-binding particles contain the magnetic material, by magnetic separation. Since the virus-binding particles used in the present invention may have particle diameters in the range of from 0.05 to 300 μm , the particles can well be centrifugally separated by means of a centrifuge.

[0066] The virus-bound particles thus separated are optionally washed with a low-concentration buffer, and thereafter normally moved to the step of separating viruses from the particles. As the method of separating viruses from the particles, a method is available in which a salt solution is made to act to dissociate viruses from the virus-bound particles. As the salt solution, a high-concentration potassium bromide, sodium bromide, 1.5 M sodium chloride, 1 mM tungstophosphoric acid solution or 1 M sodium thiocyanate may be used. The viruses thus separated from the particles are further treated according to the separation purpose. For example, the viruses are subjected to extraction of nucleic acids. Here, the extraction of nucleic acid may be made as the viruses are kept bound to the particles. For example, a buffer may be added in a small quantity to the virus-binding particles separated from the sample, followed by heating to extract the nucleic acid of viruses directly, or a commercially available extraction reagent may directly be added to the virus-bound particles to extract the nucleic acid of viruses.

- Detection of Viruses -

[0067] The method of detecting viruses according to the present invention comprises, after the step where the particles of the present invention to which the viruses have bound as described above are separated and collected from the sample, the step of;

subjecting the viruses thus separated, to a nucleic-acid amplification test.

[0068] There are no particular limitations on the nucleic-acid amplification test (NAT) of viruses. For example, usable are the PCR (polymerization chain reaction) process of F. Hoffman-La Roche Ltd., the TMA (transcription mediated amplification-hybridization protection assay) process of Gen-Probe Inc., the LCR (ligase chain reaction) process of Abbott Laboratories, the ICAN (Isothermal and chimeric primer-initiated amplification of nucleic acid) process of Takara Shuzo Co., and the LAMP (Loop-mediated isothermal amplification of DNA) process of Eiken Chemical Co.

[0069] In the virus detection method utilizing this method of separating viruses, the viruses in a specimen to be subjected to the nucleic-acid amplification test have already been separated, and hence the viruses can efficiently be detected even when the viruses contained in the original specimen or in the sample are in a very small quantity. The virus-binding particles of the present invention can also be used to detect viral proteins in a sample.

[0070] Stated specifically, a method is available in which the virus-binding particles of the present invention are mixed with a sample and the virus exosporium protein adsorbed on the particles is detected with a labeled antibody. In this method, the type of the labeled antibody may be changed to detect virus exosporium proteins of various types.

EXAMPLES

[0071] Examples of the present invention are given below. The present invention should by no means be construed limitative to these Examples. In the following "part(s)" represents part(s) by weight unless otherwise noted.

[0072] The particle diameter of virus-binding particles obtained in these Examples and the quantity of cationic groups present in the particles were measured in the following way.

- Measurement of particle diameter:

[0073] A photograph of particles was taken on an optical microscope, a scanning electron microscope or a transmission electron microscope, and particle diameters of 200 particles were measured to find their average value.

- Quantity of cationic groups present in particles:

(1) Conductivity titrimetry

[0074] The virus-binding particles were washed twice with pure water by centrifugation or magnetic separation, and a mixed-bed type ion-exchange resin was added in an amount of 5 g per 1 g of the particles. The mixture obtained was stirred for 1 hour, and thereafter the mixed-bed type ion-exchange resin was filtered off. This purification with the mixed-bed type ion-exchange resin was repeated twice. The resultant purified particles were titrated using a normal sulfuric acid solution as a titrant. This method was applied in Examples 1 to 3.

(2) Non-aqueous titrimetry

[0075] The virus-binding particles were purified in the same manner as the above (1). Thereafter, the purified product was dried and was dispersed in chloroform, and then chloroform-insoluble matter was filtered off, and then the filtrate obtained was subjected to titration using a normal solution of perchloric acid/acetic acid solution to determine the quantity of cationic groups. This method was applied in Examples 4 to 6.

- Quantity of anionic groups present in particles:

[0076] To about 10 g of the particles, 90 g of ion-exchanged water and 30 g of an anion/cation-exchange resin mixture (AMBERLITE MB3, trade name, available from Organo K.K.), and the mixture obtained was gently stirred for 1 hour. The ion-exchange resin was filtered off with a nylon mesh (48 meshes; pore size: 295 μm), and the quantity of the particles was determined, followed by conductivity titration using 0.01 mol/L of a sodium hydroxide solution to determine the quantity of anionic groups.

Example 1

[0077] Acetone was added to an oily magnetic fluid MARPO MAGNA FV 55, trade name, available from Matsumoto Yushi Seiyaku, Co., Ltd., to cause particles to undergo precipitation sedimentation, followed by drying to obtain a ferrite type superparamagnetic material having lipophilic-treated particle surfaces (particle diameter: 0.01 μm).

[0078] Then, to 40 parts (by weight; the same applies hereinafter) of the superparamagnetic material, 90 parts of cyclohexyl methacrylate, 10 parts of chloride of trimethylaminoethyl methacrylate and 3 parts of benzoyl peroxide (polymerization initiator) were added, and the resultant system was mixed and stirred to disperse the superparamagnetic material uniformly, thus a monomer composition was prepared.

[0079] Meanwhile, 10 parts of polyvinyl alcohol, 0.5 part of sodium laurate and 0.1 part of polyethylene oxide non-ylphenyl ether were dissolved in 1,000 parts of water, thus an aqueous monomer composition was prepared.

[0080] In the aqueous monomer composition thus obtained, the above monomer composition in which the superparamagnetic material has been dispersed, was added, and the mixture was preliminarily stirred by means of a homogenizer, followed by dispersion treatment by means of an ultrasonic dispersion machine, thus a suspension (an oil-drop dispersion) was prepared in which oil drops (oil phase) of 1 μm in average particle diameter were dispersed in the aqueous medium.

[0081] Next, the suspension thus obtained was charged into a 2-liter volume three-necked flask having a stirrer, and this system was heated to a temperature of 75°C to polymerize (suspension polymerization) the monomers in oil drops over a period of 5 hours, in an atmosphere of nitrogen and with stirring, thus virus-binding particles of the present invention were produced.

Example 2

[0082] Virus-binding particles of the present invention were produced in the same manner as in Example 1 except that 10 parts of the chloride of trimethylaminoethyl methacrylate used therein was replaced with 10 parts of dimethylaminoethyl methacrylate.

Example 3

[0083] Virus-binding particles of the present invention were produced in the same manner as in Example 1 except that the cyclohexyl methacrylate used therein was used in an amount of 100 parts, the chloride of trimethylaminoethyl methacrylate was not used and 3 parts of the benzoyl peroxide (polymerization initiator) was replaced with 5 parts of 2,2'-azobis(2-amidinopropane) dibasic acid salt.

Example 4

[0084] (1) Magnetic polymer particles were produced in the same manner as in Example 1 except that the cyclohexyl methacrylate used therein was used in an amount of 95 parts and 10 parts of the chloride of trimethylaminoethyl methacrylate was replaced with 5 parts of methacrylic acid.

[0085] (2) The magnetic polymer particles obtained in the above (1) were dispersed in an aqueous 5 mM sodium hydroxide solution, followed by treatment at 80°C for 12 hours to obtain carboxy-modified magnetic polymer particles.

[0086] (3) 1 g of the carboxy-modified magnetic polymer particles thus obtained were added to 20 mL of a 10 mM MES buffer (pH 6), and 1 mL of an aqueous 30% polyethyleneimine (number-average molecular weight: 70,000) solution and 0.2 g of a water-soluble carbodiimide reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride were further added, where reaction was carried out at 20°C for 2 hours to obtain virus-binding particles of the present invention.

[0087] (4) To the reaction mixture obtained, 5 g of mixed-bed type ion-exchange resin was added, and thereafter the mixed-bed type ion-exchange resin was removed by filtration. This purification with the mixed-bed type ion-exchange resin was repeated twice, followed by drying.

[0088] The magnetic polymer particles thus dried were dissolved in 10 mL of chloroform, and the magnetic material was separated by filtration and was subjected to non-aqueous titration with 0.01 mol/L of a perchloric acid/acetic acid solution to find that the total amino nitrogen combined with the magnetic polymer particles obtained in the above (2) was in an amount of 45.0 $\mu\text{mol/g}$.

Example 5

[0089] (1) 1 g (dry weight) of the carboxy-modified magnetic polymer particles obtained in the same manner as in Example 4, step (2), were added to 20 mL of a 10 mM MES buffer, and 1 mL of an aqueous 1% poly-L-lysine hydrogen bromide (number-average molecular weight: 300,000) and 0.2 g of a water-soluble carbodiimide reagent, EDC hydrochloride (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) were further added, where reaction was carried out at 20°C for 2 hours to obtain virus-binding particles of the present invention, comprised of poly-L-lysine-fixed magnetic particles.

[0090] (2) The poly-L-lysine-fixed magnetic particles thus obtained were purified in the same manner as in Example 4, step (4), followed by drying. The resultant particles were dissolved in 10 mL of dimethyl sulfoxide, and the magnetic material was separated by filtration, which was then subjected to non-aqueous titration with 0.01 mol/L of a perchloric acid/acetic acid solution to find that the total amino nitrogen combined with the magnetic polymer particles obtained in the above (1) was in an amount of 22.0 $\mu\text{mol/g}$.

Example 6

[0091] (1) Glycidyl-modified magnetic particles were obtained in the same manner as in Example 1 except that 10 parts of the chloride of trimethylaminoethyl methacrylate was replaced with 10 parts of glycidyl methacrylate.

[0092] (2) 1 g (dry weight) of the glycidyl-modified magnetic particles obtained were suspended in 20 mL of distilled water containing 1% of pyridine, and 1 mL of an aqueous 30% polyethyleneimine (number-average molecular weight: 70,000) solution was further added, where reaction was carried out at 60°C for 24 hours to obtain virus-binding particles of the present invention, comprised of polyethyleneimine-fixed magnetic particles.

[0093] (3) The polyethyleneimine-fixed magnetic particles thus obtained were purified in the same manner as in Example 4, step (4), followed by drying. The resultant particles were dissolved in 10 mL of chloroform, and the magnetic material was separated by filtration, which was then subjected to non-aqueous titration with 0.01 mol/L of a perchloric acid/acetic acid solution to find that the total amino nitrogen combined with the magnetic particles obtained in the above (2) was in an amount of 20 $\mu\text{mol/g}$.

Table 1

	Particle diameter (μm)	Cationic-group quantity (mol/g)
Example 1	1.2	5.4×10^{-7}
Example 2	3.7	7.8×10^{-8}
Example 3	1.1	5.0×10^{-7}
Example 4	0.7	4.5×10^{-6}
Example 5	0.8	1.1×10^{-6}

EP 1 108 743 A2

Table 1 (continued)

	Particle diameter (μm)	Cationic-group quantity (mol/g)
Example 6	1.2	1.0×10^{-5}

Example 7 (Test 1)

[0094] (1) Aqueous dispersions of the virus-binding particles obtained in Examples 1 to 6 were purified and thereafter the concentration of the particle solid-matter was adjusted to 5% using physiological saline solution (Experiment Nos. 1 to 6).

[0095] An aqueous dispersion of the virus-binding particles obtained in Example 2 was also purified and thereafter the concentration of the particle solid-matter was adjusted to 5% by weight using physiological saline solution, and then manganese dichloride was added so that the concentration thereof might become 100 mmol/L (Experiment No. 7).

[0096] (2) To 1 mL of human plasma containing 10^4 copies/mL of HBV (hepatitis B virus), 100 μL of the particle suspension (5% by weight) obtained in the above (1) was added, followed by rotational stirring at room temperature for 10 minutes.

[0097] After the reaction was completed, the mixture was set on a magnetic separation stand to effect separation into particles and a supernatant, where the supernatant was discarded to obtain the particles. To the particles obtained in this way, 50 μL of an aqueous 1 M sodium thiocyanate was added, and the mixture obtained was stirred for 5 minutes, which was thereafter separated into particles and a supernatant on a magnetic separation stand to obtain the supernatant (separated fraction). Its final volume was about 50 μL .

[0098] The separated fraction supernatant was taken to effect nucleic-acid extraction by a conventional method, and the quantity of DNA was determined by the TaqMan PCR process, using ABI PRISM TM7700 Sequence Detection System (made by Perkin Elmer Applied Biosystems Corp.). To make the determination of DNA, in the course of amplification made 40 times, the number of cycles (Th cycles) where fluorescence intensity exceeded a stated reference value (threshold: Th) was extrapolated on a calibration curve of DNA quantity Th cycles which was prepared by making measurement simultaneously, using a dilution series of a specimen whose virus concentration was known.

[0099] The results obtained are shown in Table 2.

Comparative Example 1

[0100] 10 μL of a heparin solution (prepared by dissolving 160/USP units/mg of heparin in 5 mL of a 0.15 M sodium chloride solution) and 75 μL of 1 M MnCl_2 were added to 1 mL of human plasma containing 10^4 copies/mL of HBV, followed by rotational stirring at room temperature for 20 minutes.

[0101] After the reaction was completed, the mixture was separated at 15,000 rpm for 10 minutes by means of a low-speed microcentrifuge, and the supernatant formed was discarded to obtain a sediment. To the sediment obtained in this way, 50 μL of saturated potassium bromide was added to solubilize the sediment (separated fraction). Its final volume was about 50 μL .

[0102] The separated fraction was taken to effect nucleic-acid extraction by a conventional method, and the quantity of DNA was determined by the TaqMan PCR process, using ABI PRISM TM7700 Sequence Detection System (made by Perkin Elmer Applied Biosystems Corp.). To make the determination of DNA, in the course of amplification made 40 times, the number of cycles (Th cycles) where fluorescence intensity exceeded a stated reference value (threshold: Th) was extrapolated on a calibration curve of DNA quantity Th cycles which was prepared by making measurement simultaneously, using a dilution series of a specimen whose virus concentration was known.

[0103] The results obtained are shown in Table 2.

Table 2

Experiment No.	Type of particles	Amount of 1M MnCl_2	Total collected DNA quantity (copies)	Collection rate (%)	Concen- trating rate (times)
1	Example 1	0	6.78×10^6	67.8	6.8
2	Example 2	0	5.90×10^6	59.0	5.9
3	Example 3	0	8.30×10^6	83.0	8.3
4	Example 4	0	1.06×10^7	100	10

Table 2 (continued)

Experiment No.	Type of particles	Amount of 1M MnCl ₂	Total collected DNA quantity (copies)	Collection rate (%)	Concen- trating rate (times)
5	Example 5	0	1.03 X 10 ⁷	100	10
6	Example 6	0	1.10 X 10 ⁷	100	10
7	Example 2	100	9.82 X 10 ⁶	98.2	9.8
10	Comparative Example 1	75	-	0	-

Example 8

[0104] An aqueous dispersion of the virus-binding particles obtained in Example 2 was purified and thereafter its solid-matter concentration was adjusted to 5% using physiological saline solution, and 100 μ L of this dispersion was added to 100 μ L of a dilute sample fraction of an HBsAg positive specimen whose concentration was known, followed by stirring for 15 minutes by means of a tube mixer. After the stirring was completed, particles were separated on a magnetic separation stand. The supernatant formed was removed, and subsequently the particles were washed with tris buffer physiological saline solution (TBS) of pH 7.5, where 100 μ L of a peroxidase labeled HBs antibody (available from Tokushu Men-eki Kenkyusho) was added to carry out reaction. The reaction mixture was washed with TBS, and 100 μ L of a substrate solution was added to effect color development reaction for 30 minutes. Then, sulfuric acid was added to terminate the reaction, and the absorbance at each concentration was measured.

[0105] Results obtained are shown in Table 3.

Table 3

HBs concentration (ng/mL)	0	0.1	0.3	1.0	3.0	10.0	30.0
Absorbance	0.07	0.14	0.21	0.98	1.84	2.30	2.30

Example 9

[0106] Preparation of surface-sulfonated styrene/divinylbenzene copolymer particles:

To 10 g of styrene/divinylbenzene copolymer particles (trade name: IXP-SS-3-TXG/57; available from Dyno K.K.; molar ratio of styrene/divinylbenzene: 97/3; particle diameter: 3 μ m), 100 g of concentrated sulfuric acid (98% by weight) was added, and reaction was carried out at 30°C for 5 hours. After the reaction was completed, particles were washed with pure water and then with phosphoric acid buffer physiological saline solution (hereinafter "PBS") to obtain sulfonated styrene/divinylbenzene copolymer particles.

Their particle diameter and the quantity of anionic groups present in particles are shown in Table 4.

Example 10

[0107] The particles obtained in the step (1) of Example 4 were dispersed in an aqueous 5 mM sodium hydroxide solution, followed by treatment at 80°C for 12 hours to obtain carboxyl-containing particles.

[0108] Their particle diameter and the quantity of anionic groups present in particles are shown in Table 4.

Example 11

[0109] Preparation of sulfonic acid monomer seed polymerization particles:

8 g of divinylbenzene polymer particles (trade name: N60801; available from Dyno K.K.; 55%-purity divinylbenzene polymer; particle diameter: 9.8 μ m) were dispersed in 100 g of water containing 0.03 g of sodium dodecylsulfate. To the dispersion obtained, 1 g of styrene and 1 g of sodium styrenesulfonate were added, and the mixture was heated to 80°C, and thereafter 0.01 g of potassium persulfate was added to carry out seed-polymerization for 10 hours. After the reaction was completed, particles were washed with pure water and then with PBS to obtain styrenesulfonic acid/styrene copolymer particles.

Their particle diameter and the quantity of anionic groups present in particles are shown in Table 4.

Example 12

[0110] Preparation of cross-linked isoprenesulfonic acid gel particles:

8.5 g of isoprenesulfonic acid, 1.4 g of N,N'-methylenebisacrylamide and 0.1 g of potassium persulfate were dissolved in 20 g of water. To the solution formed, 15 g of iso-octane to which 0.5 g of sorbitan monostearate (Trade-name: Span 60, supplied by Wako Pure Chemical Industries, Ltd.) had been added was added. The mixture obtained was vigorously stirred and was thereafter heated to 80°C to carry out reaction for 5 hours. After the reaction was completed, particles were washed with pure water and then with PBS to obtain cross-linked isoprenesulfonic acid gel particles.

Their particle diameter and the quantity of anionic groups present in particles are shown in Table 4.

Example 13

[0111] Preparation of polystyrenesulfonic acid-fixed particles:

35 g of styrene, 0.44 g of n-butyl lithium and 200 g of cyclohexane were charged into a pressure reaction vessel, and reaction was carried out for 4 hours, keeping temperature at 60 to 90°C. Thereafter, carbon dioxide was blown into the reaction vessel to terminate the reaction. Then, the solvent and unreacted monomers were evaporated off under reduced pressure, and thereafter 100 g of ether was added to make dilution to obtain a carboxyl-terminated polystyrene solution. To this polymer solution, 41 g of concentrated sulfuric acid was little by little added, and the mixture formed was continued to be stirred at 50°C to proceed sulfonation for 5 hours. Thereafter, the solvent was evaporated off under reduced pressure, and the product obtained was neutralized with sodium hydroxide, followed by purification by dialysis to obtain a sodium carboxyl-terminated polystyrenesulfonate (molecular weight: 26,000).

Next, 10 g of the particles obtained at the step (1) of Example 10 were dispersed in 50 g of water, and 0.1 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was added, where reaction was carried out at 4°C for 1 hour. Thereafter, the reaction mixture was washed with cold water, and then 0.5 g of hexamethylenediamine was added, where reaction was carried out at room temperature for 2 hours, followed by washing with pure water to obtain hexamethylenediamine-fixed particles. To the particles thus obtained, 0.1 g of EDC and 0.5 g of the sodium carboxyl-terminated polystyrenesulfonate obtained above were added, and reaction was carried out at room temperature for 2 hours. Thereafter, the particles were washed with pure water and then with PBS to obtain sodium polystyrenesulfonate-fixed particles.

Their particle diameter and the quantity of anionic groups present in particles are shown in Table 4.

- Test 2:

[0112] Concentrating of virus and determination of nucleic acid:

(1) Using the particles obtained in the above Examples 9 to 13, viruses were concentrated. To 1 mL of human plasma containing 3×10^4 copies/mL of HCV, 100 μ L each of corresponding particle suspensions (10% by weight) and 25 μ L of 1 M manganese dichloride were added, followed by rotational stirring at room temperature for 10 minutes. After the reaction was completed, the mixture was separated by means of a refrigerated microcentrifuge. The supernatant formed was discarded to obtain particles. To the particles obtained in this way, 50 μ L of saturated sodium bromide was added, and the mixture obtained was stirred for 5 minutes, which was thereafter separated at 15,000 rpm for 1 minute by means of the refrigerated microcentrifuge to obtain a supernatant (separated concentrated fraction). Its final volume was about 50 μ L.

(2) The separated fraction was taken to effect nucleic-acid extraction by a conventional method, and the quantity of RNA was determined by the TaqMan RT-PCR process, using ABI PRISM TM7700 Sequence Detection System (made by Perkin Elmer Applied Biosystems Corp.). To make the determination of RNA, in the course of amplification made 40 times, the number of cycles (Th cycles) where fluorescence intensity exceeded a stated reference value (threshold: Th) was extrapolated on a calibration curve of RNA quantity Th cycles which was prepared by making measurement simultaneously, using a dilution series of a specimen whose virus concentration was known.

Comparative Example 2

[0113] To 1 mL of the same human plasma as that used in Test 2 noted above, 10 μ L of a heparin solution (prepared by dissolving 160/USP units/mg of heparin in 5 mL of a 0.15 M sodium chloride solution) was added instead of virus-binding particles according to the present invention, and 75 μ L of 1 M $MnCl_2$ was further added to carry out reaction at room temperature for 20 minutes. After the reaction was completed, the mixture was separated at 15,000 rpm for 10 minutes by means of a low-speed microcentrifuge, and the supernatant formed was discarded to obtain a sediment. To this sediment, 50 μ L of saturated potassium bromide was added to solubilize the sediment (separated concentrated fraction). Its final volume was about 50 μ L. Thereafter, evaluation was made in the same manner as in (2) of Test 2.

[0114] Collected quantity, concentrating rate and time taken to obtain a separated and concentrated fraction are shown in Table 4.

[0115] As shown in Table 4, in the method of Comparative Example 2, any nucleic acid was not detectable because of PCR inhibition due to heparin. On the other hand, in all Examples, the rate of virus collection was substantially 100%, and the concentrating rate was as high as 25 times.

Table 4

Example	Particle diameter (μ m)	Anionic-group quantity (mol/g)	Sample, volume	HCV-RNA quantity	Collection rate (%)	Concentrating rate (times)	Time taken (min.)
Before collection by separation			1 mL	3×10^4			
After collection by separation							
Example 9	3	8.4×10^{-8}	50 μ L	3.2×10^4	About 100%	25	35
Example 10	0.71	2.1×10^{-4}	50 μ L	2.8×10^4	About 100%	25	38
Example 11	9.8	1.5×10^{-8}	50 μ L	2.6×10^4	About 100%	25	34
Example 12	85	7.8×10^{-3}	50 μ L	3.8×10^4	About 100%	25	34
Example 13	0.7	5.6×10^{-7}	50 μ L	3.6×10^4	About 100%	25	35
Comparative Example 2			50 μ L	Undetectable	0%	-	40

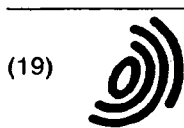
[0116] As described above, the use of the virus-binding particles of the present invention enables simultaneous treatment of a large number of specimens with ease to separate and concentrate viruses by a simple means of making centrifugation or making magnetism act. Besides, the resultant samples containing separated concentrated viruses do not adversely affect the treatment for nucleic-acid amplification. The method of separating viruses by using such particles enables viruses to be separated in a good efficiency and in a short time, from specimens containing viruses in a very small quantity. Also, the method of detecting viruses enables detection of viruses at a high precision.

[0117] The virus-binding particles of the present invention, having adsorbed viruses thereon from specimens, can also be used for immunological assay.

[0118] Particles capable of being bound by viruses, having a particle diameter of 0.05 μ m to 300 μ m and having either a cationic group or an anionic group or both at their surfaces. Also disclosed are a method of separating or detecting viruses and a virus-separating reagent, which make use of such particles. The particles enable simultaneous and simple treatment of a large number of specimens by a simple means.

Claims

1. Particles capable of being bound by viruses, having a particle diameter of 0.05 μm to 300 μm and having either a cationic group or an anionic group or both at their surfaces.
2. The particles capable of being bound by viruses according to claim 1, wherein said cationic group is an amino group, a quaternary ammonium group, an imino group, an amidino group, a hydrazino group, or a pyridyl group.
3. The particles capable of being bound by viruses according to claim 1, wherein said anionic group is a carboxyl group or a sulfonic acid group.
4. The particles capable of being bound by viruses according to any one of claims 1 to 3, wherein said particles comprise a magnetic material.
5. A virus-separating reagent comprising particles according to any one of claims 1 to 4, and a polyvalent metal compound.
6. A method of separating viruses, comprising the steps of: adding the particles according to any one of claims 1 to 4, to a sample which possibly contains viruses, to allow the viruses to bind to the particles; and separating from the sample the particles to which the viruses have bound to collect the particles.
7. The method of separating viruses according to claim 6, wherein a polyvalent metal compound is added to said sample together with said particles.
8. A method of detecting viruses, comprising the steps of:
 - adding the particles according to any one of claims 1 to 4, to a sample which possibly contains viruses, to allow the viruses to bind to the particles;
 - separating viruses from the sample by separating from the sample the particles to which the viruses have bound, to collect the viruses; and
 - subjecting the viruses thus separated, to a nucleic-acid amplification test.
9. The method of detecting viruses according to claim 8, wherein a polyvalent metal compound is added to said sample together with said particles.
10. A method of detecting viruses, comprising the steps of: adding the particles according to any one of claims 1 to 4, to a sample which possibly contains viruses, to allow the viruses to bind to the particles; separating viruses from the sample by separating from the sample the particles to which the viruses have bound; and subjecting the viruses bound to the particles, to an immunological assay.
11. The method of detecting viruses according to claim 10, wherein a polyvalent metal compound is added to said sample together with said particles.
12. Use of the particles according to any one of claims 1 to 4 in separation of viruses from a sample which possibly contains viruses:
13. Use of the particles according to any one of claims 1 to 4 in detection of viruses present in a sample.



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 108 743 A3**

(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:
06.02.2002 Bulletin 2002/06

(51) Int Cl.7: **C08K 3/00**, C08K 3/22,
C12N 7/00, C12N 7/02,
B03C 1/01

(43) Date of publication A2:
20.06.2001 Bulletin 2001/25

(21) Application number: **00126977.8**

(22) Date of filing: **08.12.2000**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **08.12.1999 JP 34904199**
16.02.2000 JP 2000038010

(71) Applicant: **JSR Corporation**
Tokyo (JP)

(72) Inventors:
• **Sato, Kouei**
Sashima-gun, Ibaraki-ken (JP)

- **Tanaka, Takeshi**
Tokyo (JP)
- **Murata, Mitsuhiro**
Ushiku-shi, Ibaraki-ken (JP)
- **Nishida, Shozou**
Iizuka-shi, Fukuoka-ken (JP)
- **Hikata, Mikio**
Tsuchiura-shi, Ibaraki-ken (JP)
- **Kasai, Kiyoshi**
Kameyama-shi, Mie-ken (JP)

(74) Representative:
Leson, Thomas Johannes Alois, Dipl.-Ing. et al
Patentanwälte Tiedtke-Bühling-Kinne & Partner,
Bavariaring 4
80336 München (DE)

(54) **Virus-binding particles, virus-separating reagent, separation of viruses, and detection of viruses**

(57) Particles capable of being bound by viruses, having a particle diameter of 0.05 μm to 300 μm and having either a cationic group or an anionic group or both at their surfaces. Also disclosed are a method of separating or detecting viruses and a virus-separating

reagent, which make use of such particles. The particles enable simultaneous and simple treatment of a large number of specimens by a simple means.

EP 1 108 743 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 12 6977

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 99 35500 A (BIO MERIEUX ;ELAISSARI ABDELHAMID (FR); MANDRAND BERNARD (FR); PIC) 15 July 1999 (1999-07-15) * claims *	1-13	C08K3/00 C08K3/22 C12N7/00 C12N7/02 B03C1/01
X	WO 97 00896 A (VASLIN SOPHIE ;PROLABO SA (FR); RICHARD JOEL (FR)) 9 January 1997 (1997-01-09) * claims *	1-13	
P,X	WO 00 42432 A (LABSYSTEMS OY ;PALOMAEKI PEKKA (FI); TUUNANEN JUKKA (FI); KAERMENI) 20 July 2000 (2000-07-20) * page 3, line 1 - line 10; claims 1-11 *	1-13	
X	EP 0 709 680 A (FUJIREBIO KK) 1 May 1996 (1996-05-01) * claims *	1-13	
X	FR 2 463 807 A (PASTEUR INSTITUT) 27 February 1981 (1981-02-27) * page 2, line 19 - page 3, line 36 *	1-13	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
X	DE 198 00 294 A (MUELLER SCHULTE DETLEF DR) 8 July 1999 (1999-07-08) * column 7, line 57 - line 63; claims *	1-13	C08K C12N B03C
P,X	PATENT ABSTRACTS OF JAPAN vol. 2000, no. 14, 5 March 2001 (2001-03-05) & JP 2000 306718 A (JSR CORP), 2 November 2000 (2000-11-02) * abstract *	1-13	
A	WO 97 11160 A (LIGOCHEM INC ;KRUPPEY JOHN (US); SMITH ALLEN D (US); ARNOLD EDWARD) 27 March 1997 (1997-03-27) * claims *	1-3,6-13	
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 10 December 2001	Examiner Andriollo, G
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : technological background O : non-written disclosure P : intermediate document & : member of the same patent family, coresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category			

EPO FORM 1503 01/02 (P/C/C1)

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 00 12 6977

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

10-12-2001

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 9935500	A	15-07-1999	FR	2773416 A1	09-07-1999
			AU	1973499 A	26-07-1999
			EP	1046037 A1	25-10-2000
			WO	9935500 A1	15-07-1999
WO 9700896	A	09-01-1997	FR	2735778 A1	27-12-1996
			AT	200901 T	15-05-2001
			CA	2198250 A1	09-01-1997
			DE	69612654 D1	07-06-2001
			DE	69612654 T2	08-11-2001
			DK	777691 T3	24-09-2001
			EP	0777691 A1	11-06-1997
			ES	2157448 T3	16-08-2001
			WO	9700896 A1	09-01-1997
			JP	10505630 T	02-06-1998
			NO	970792 A	07-04-1997
			US	5976426 A	02-11-1999
WO 0042432	A	20-07-2000	EP	1145010 A1	17-10-2001
			WO	0042432 A1	20-07-2000
			NO	20013541 A	17-09-2001
EP 0709680	A	01-05-1996	AU	681893 B2	11-09-1997
			AU	7084594 A	06-02-1995
			DE	69428445 D1	31-10-2001
			EP	0709680 A1	01-05-1996
			JP	3192149 B2	23-07-2001
			WO	9502185 A1	19-01-1995
FR 2463807	A	27-02-1981	FR	2463807 A1	27-02-1981
DE 19800294	A	08-07-1999	DE	19800294 A1	08-07-1999
JP 2000306718	A	02-11-2000	NONE		
WO 9711160	A	27-03-1997	US	5658779 A	19-08-1997
			AU	7376396 A	09-04-1997
			CA	2232622 A1	27-03-1997
			EP	0857204 A1	12-08-1998
			JP	11511335 T	05-10-1999
			WO	9711160 A1	27-03-1997

EPO FORM P0439

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82